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# The role of microtubules in electrotaxis of rat Walker carcinosarcoma WC256 cells*

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**The endogenous electric field may provide an important signal for directional cell migration during cancer metastasis but the mechanism of cell electrotaxis is poorly understood. It was postulated that microtubules play a central role in the polarization and directional migration of several types of cells. In this paper we investigated the role of microtubules in electrotaxis of rat Walker carcinosarcoma WC256 cells. We found that colchicine-stimulated disassembly of microtubules caused the formation of blebs instead of lamellipodia at the front of about 45% of cells. Most of the remaining cells contracted and became rounded or transformed into non-polar cells. Depolymerization of microtubules in both subpopulations of cells reduced the directionality of cell migration to about 50% of the control, but bleb- forming cells migrated much more efficiently than lamellipodia-forming cells. The analysis of microtubules architecture in the presence of an endogenous electric field showed that there is no relationship between the direction of migration and the polarization of microtubules. These results suggest that microtubules are not indispensable for electrotaxis of WC256 cells, however they may improve the directionality of cell migration.**

**Key words**: rat Walker carcinosarcoma WC256 cells, electrotaxis, directional cell migration, microtubules

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## INTRODUCTION

Directional cell migration requires a distinct polarity, generated by an integrated crosstalk of signaling molecules, signaling pathways, cytoskeleton, and adhesion (Ridley *et al.,* 2003; Petrie *et al.,* 2009; Lara Rodriguez & Schneider, 2013). Microtubules (MTs) were proposed to play a central role in the development and/or maintenance of cell polarity in a cell type-dependent manner (Small *et al.,* 1999a; Small & Kaverina, 2003; Vinogradova *et al.,* 2009; Kaverina & Straube, 2011). Microtubule disassembly in strongly adherent cells such as fibroblasts and macrophages (Vasiliev *et al.,* 1970; Bershadsky *et al.,* 1991; Glasgow & Daniele, 1994; Small *et al.,* 1999b) impairs their polarity and migration, whereas it promotes motility of neutrophils but impairs their directionality (Xu *et al.,* 2005). In contrast, polarity and motility are independent of microtubules in fish keratinocytes (Etienne-Manneville, 2004), primary cultures of fibroblasts (Middleton *et al.,* 1989) and *Dictyostelium discoideum* cells (Sroka *et al.,* 2002a).

Cells can migrate directionally in response to several chemical and physical factors, including an electric field. The endogenous electric field (EF) may provide an important signal for directional cell migration during wound healing, embryonic development and cancer metastasis (Djamgoz *et al.,* 2001; Mycielska & Djamgoz, 2004; McCaig *et al.,* 2005; Zhao, 2009). Many cell types respond to applied direct current electric field (dcEF). Most cells such as human keratinocytes (Sheridan *et al.,* 1996), fibroblasts (Kim *et al.,* 2015), highly metastatic rat prostate MAT-LyLu cancer cells (Djamgoz *et al.,* 2001) and fish epidermal cells (Cooper & Shliwa, 1986) migrate towards the cathode. However, human granulocytes (Rapp *et al.,* 1988), rabbit corneal endothelial cells (Chang *et al.,* 1996), human vascular endothelial cells (HUVECs) (Zhao *et al.,* 2004) and the weakly metastatic rat prostate AT-2 cancer cells (Djamgoz *et al.,* 2001) show anodal response.

Although the phenomenon of electrotaxis has been well documented for a variety of cells, the mechanism of directional response of cells to dcEF is largely unknown. Contradictory data on the role of microtubules in electrotaxis have been published making this topic controversial (Cooper & Schliwa, 1985; Finkelstein *et al.,* 2003; Rajnicek *et al.,* 2006; Sun *et al.,* 2013). Therefore, in this study we examined the function of microtubules in the polarization and directional movement of rat Walker carcinosarcoma WC256 cells exposed to dcEF.

## MATERIALS AND METHODS

**Cell culture**. The adherent subline of Walker carcinosarcoma WC256 cells was obtained by continuous culture of cells initially growing in a suspension as described previously (Sroka *et al.,* 2002b). Cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin and 10 mg/ml streptomycin in a humidified atmosphere with 5% $CO_2$ at 37°C.

**Exposition of WC256 cells to direct current electric field (dcEF)**. WC 256 cells were exposed to dcEFs of 3 V/cm in a plexiglass apparatus described in details by Korohoda *et al.* (2000). Briefly, dcEFs were applied for a specified time, up to 2.5 hours, through Ag/AgCl reversible electrodes of 6 $cm^2$ immersed in saline-filled wells connected by agar bridges (2% agar in 0.5 n KCl, 8 cm long) to neighboring wells, to which the observation chambers were attached. The observation chambers were made of cover glasses measuring: $60 \times 35 \times 0.2$ mm.

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**Abbreviations**: FCS-fetal calf serum; EF-electric field; dcEF-direct current electric field; MTs-microtubules; MTOC-microtubule organizing center; SEM-scanning electron microscopy

The investigated cells were plated for 2 hours into the chamber at a density 35000 cells/cm², and incubated in RPMI-1640 supplemented with 5% FCS in a humidified atmosphere with 5% $CO_2$ at 37°C. Then the chamber was mounted with silicone grease in the plexiglass apparatus.

**Time-lapse monitoring of movement of individual cells**. The movement of WC256 cells under isotropic conditions and in dcEF of 3 V/cm was time-lapse recorded for 2.5 hours at time intervals of 2.5 minutes. The tracks of individual cells were determined from the series of changes in the cell centroid positions, pooled and analyzed as previously described (Sroka *et al.,* 2004). The following parameters were estimated: (i) the total length of cell displacement (μm), i.e. the distance from the starting point directly to the cell's final position, (ii) the speed of cell movement (μm/min), i.e. total length of cell trajectory/time of recording, (iii) average directional cosines γ; γ is defined as the angle between the 0X axis (parallel to the field direction) and a vector AB. A and B are the first and subsequent position of the cell, respectively. The parameter equals 1 for a cell moving towards the cathode, –1 for the cell moving in the direction of the anode, and 0 for random movement (Friedl *et al.,* 1993; Korohoda *et al.,* 1997; Korohoda *et al.,* 2002, Sroka *et al.,* 2004). In some experiments WC256 cells were pre-incubated in RPMI-1640 with 5% FCS supplemented with 1 μM colchicine for 30 minutes followed by time-lapsing under isotropic conditions or in dcEF of 3 V/cm, as described above. Cell trajectories from no less than three independent experiments (number of cells = 50) were taken for the estimation of statistical significance by the t-student test; $p<0.05$.

**Wound Healing Assay (Scratch Assay)**. The cells were seeded onto a cover slide 2 hours before the experiment at density 750000 cells/cm² to form a confluent monolayer. Then scratches were performed using a 100 μl tip. The culture medium was replaced with fresh medium and the electrotactic chamber was mounted. Cell movement was recorded as described above.

**SEM analysis**. The morphology of WC256 cells under control conditions and after treatment with 1 μM colchicine for 30 minutes was investigated using SEM. The cells were fixed with a 2.5% glutaric aldehyde in a 0.1 M cacodylic buffer, rinsed with 0.1 M cacodylic buffer several times, subjected to osmium post-fixation for 1 h, and then washed 3 times in 0.1 M cacodylic buffer. The samples were subsequently dehydrated in increasing concentrations of ethanol (50–100%), rinsed with 100% acetone, dried at the critical point (− 200°C for 40 min) and left overnight under vacuum. The dehydrated samples were coated with a thin film of gold (JFC-1100F, Tokyo, Japan) and observed by SEM (JEOL JSM-5410, Tokyo, Japan) (Sowa *et al.,* 2015).

**Immunofluorescence**. WC256 cells were placed on coverslips and incubated in RPMI-1640 with 5% FCS for 2 hours. The incubation was continued for 30 minutes with or without 1 μM colchicine in isotropic conditions or in the presence of dcEF (3 V/cm). Then cells were fixed by addition of 7.4% formaldehyde (final concentration 3.7%) for 15 minutes at room temperature. After washing 3 times with PBS, cells were permeabilized by incubation in 0.1% Triton X-100 solution for 15 minutes, washed in PBS, and incubated with 1% BSA in PBS for 10 minutes. The cells were then incubated with mouse monoclonal anti-α-tubulin antibody (diluted 1:500) for 1 hour at room temperature. Cells were washed 5 times with PBS, and incubated with Alexa Fluor 488 goat anti-mouse IgG (diluted 1:300) for 45 minutes at room temperature. Fluorescent images were taken with a Leica DM IRE2 microscope.

## RESULTS AND DISCUSSION

Several reports have demonstrated the directional movement of many types of normal and cancer cells in response to dcEF (Djamgoz *et al.,* 2001; Mycielska & Djamgoz, 2004; McCaig *et al.,* 2005; Zhao, 2009). In our study we examined the effect of dcEF (3 V/cm) on the migration of WC256 cells using two independent methods i.e. the "single cell migration" assay and the "wound healing" assay. The trajectories of cells moving in the control conditions and in the presence of dcEF are shown in the circular diagrams in Fig. 1 (C, D, E, F). The quantitative data obtained are summarized in Fig. 4 (B, C, D). The analysis of individual tracks showed that in the absence of any electric stimulus, sparse cells analyzed by the "single cell migration" assay moved in all directions with the same probability ($\cos\gamma = 0.008 \pm 0.09$), whereas after application of a dcEF their movement became strongly directed towards the cathode ($\cos\gamma = 0.87 \pm 0.02$) although cells did not align parallel to the direction of movement (Fig. 1B). Additionally, those cells were more spread and elongated than control cells (Fig. 1A, 2A *vs* Fig. 1B, 2B). We also observed that the application of dcEF resulted in a double increase of cell displacement, primarily due to considerable straightening of the trajectories of movement as compared to the control (Fig. 1C, 1D; 4C). To confirm these results we used a "wound healing" assay, which is a straightforward method of studying cell migration *in vitro* and mimics to some extent migration of cells *in vivo*. We found that under isotropic conditions cells on the edges of the newly created gap moved directionally towards the opening space to close the scratch (Fig. 1E). In these experiments the average value of directional cosine γ equaled — $0.08 \pm 0.09$, because cells moved both from the right and the left edge of the scratch in opposite directions (Fig. 4D). When cells in a "wound healing" assay were exposed to dcEF they showed cathodal response to electric field ($\cos\gamma = 0.79 \pm 0.04$) (Fig. 1F; 4D). The results of the present study showed for the first time that carcinosarcoma cells can be electrotactic.

Even though the cell electrotaxis is involved in a number of basic biological processes the mechanism of this phenomenon is still poorly understood. However, several molecules and signalling pathways were suggested to be involved in the detection of physiological EF and induction of directional migration of cells, i.e. $Ca^{2+}$ signalling (Mycielska & Djamgoz, 2004), asymmetry in the distribution of growth factor receptors such as EGF (epidermal growth factor) and detergent-insoluble membrane lipids, signalling through EGF receptors/ERK1/2, through integrin/Rac, cAMP/PKA, reactive oxygen species (ROS) and many others (Zhao *et al.,* 1999; Zhao *et al.,* 2002; Pullar *et al.,* 2006; Li *et al.*, 2012). The reorganization of the cytoskeletal structures, including actin and MTs has an unquestionable role in establishing and maintaining the polarity and directionality of migration of most types of cells. While the asymmetric polarization of F-actin was observed in dcEF stimulated cells (Chang *et al.*, 1996; Pu & Zhao, 2005; Yan *et al.*, 2009; Li *et al.*, 2012), little is known about the role of MTs in cellular electrotaxis. Moreover, these data are contradictory. It was reported that growth cones steering by a physiological field requires dynamic microtubules (Rajnicek *et al.,* 2006), while disruption of microtubules does not

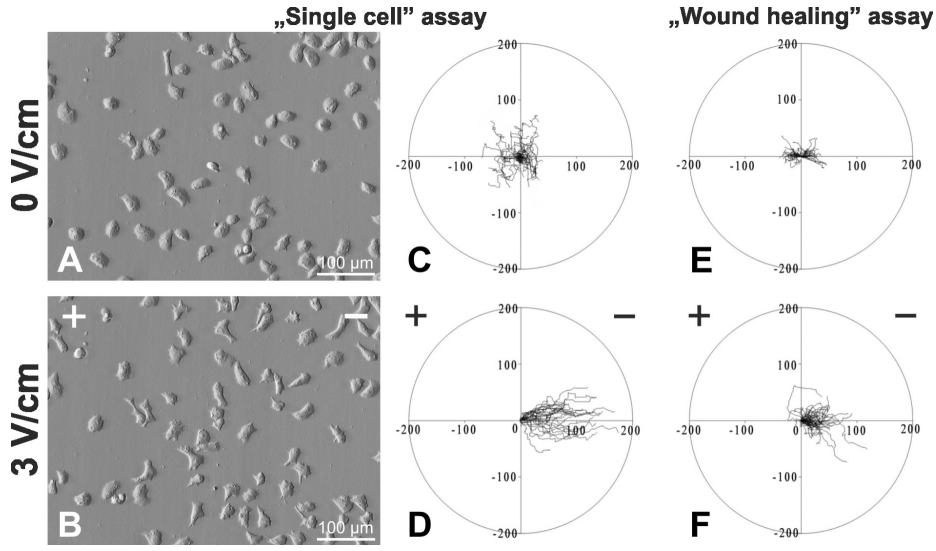


**Figure 1. The effect of dcEF on the morphology and migration of WC256 cells.**
Cell morphology under isotropic conditions (**A**) and in the presence of dcEF (3 V/cm) (**B**). Composite trajectories of cells migrating in the absence (**C, E**) and in the presence of dcEF (**D, F**) studied with a "single cell" (**C, D**) and "wound healing" (**E, F**) assays. At circular diagrams, the initial point of each trajectory was placed at the center of the circle and the x-axis corresponds to the direction of electric field. The cathode was placed always at the right side of the diagram and each trajectory was constructed from 60 successive positions of cell centroid recorded at 2.5 min. time intervals, immediately after the exposure of cells to dcEF. The analysis of individual tracks showed that after application of dcEF cell movement became strongly directed towards the cathode. Scale in µm.

affect keratinocyte, keratinocyte fragments or fibroblast electrotaxis (Finkelstein *et al.,* 2003; Cooper & Schliwa, 1985; Sun *et al.,* 2013). Therefore, in the next series of experiments we analyzed the microtubule polarization in WC256 cells exposed to dcEF. We tested the hypothesis that MTs or microtubule organizing center (MTOC) redistribution towards the leading edge is required for a directional reaction of WC256 cells. The locations at which microtubules nucleate and spread out in polarized cells moving directionally depend on the cell type. In fibroblasts but not in epithelial cells microtubules nucleate from the MTOC at the front of cells and extend to the leading edge of cells (Yvon *et al.,* 2002). On the contrary, in leukocytes the MTOC can be localized at the front (Schliwa *et al.,* 1982) or at the rear of the cells (Xu *et al.,* 2005).

In the present study, immunofluorescent staining of MTs in WC256 cells showed that neither dcEF nor "wounding" altered the distribution of the MTOC or MTs as compared to the control; these structures remained non-polarized in the direction of the electric field or scratch (Fig. 2). To verify that MTs do not play

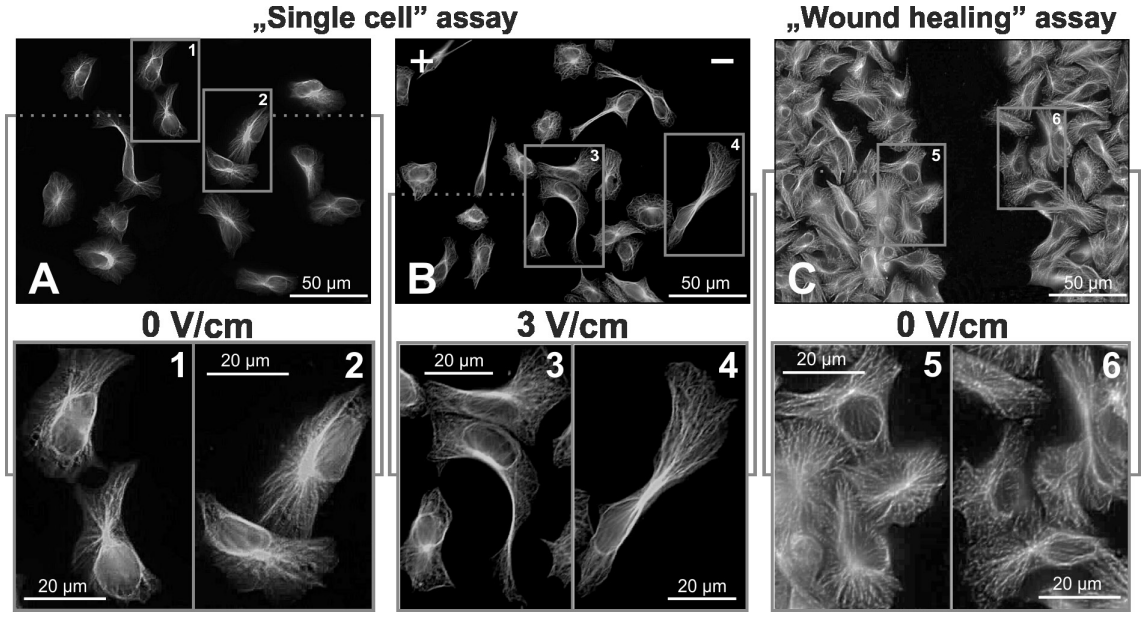


**Figure 2. The effect of dcEF on the organization of microtubules in WC256 cells.**
(**A, B**) a "single cell" assay, control (**A**), 3 V/cm (**B**); (**C**) a "wound healing" assay. Cells were fixed, stained with mouse monoclonal anti-α-tubulin antibody and counterstained with secondary antibody conjugated with Alexa Fluor 488 dye. Immunofluorescent staining showed that neither dcEF nor "wounding" altered the distribution of the MTOC and MTs as compared to control and still remained non-polarized in the direction of electric field or scratch. Inserts show an enlarged regions marked in images.

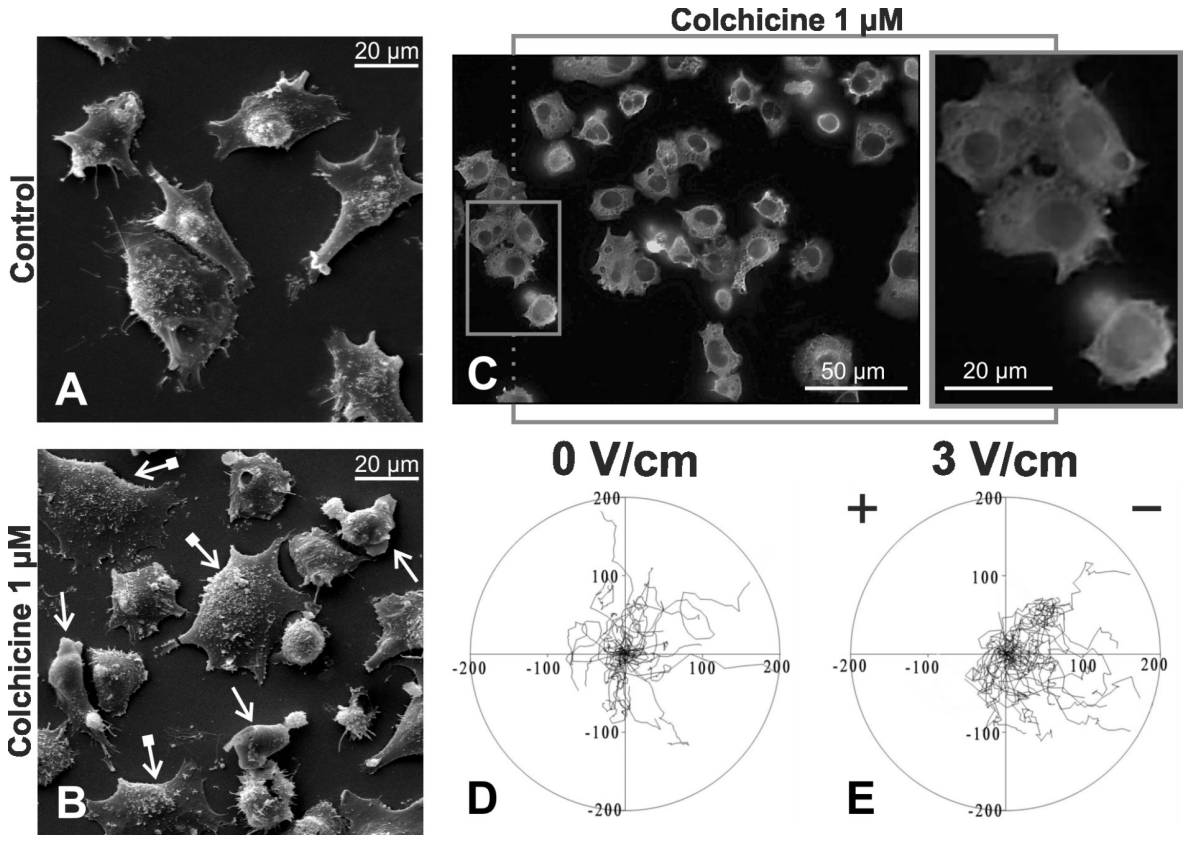


**Figure 3. The effect of colchicine on the morphology, organization of microtubules and migration of WC256 cells.**
(**A, B**) SEM pictures of untreated and colchicine-treated WC256 cells. Cells were incubated in medium without (**A**) or with 1 μM colchicine (**B**) for 30 minutes at 37°C, fixed and further proceeded for SEM analysis. Analysis revealed the existence of two subpopulations of colchicine-treated WC256 cells, i.e. blebbing, fast migrating cells (straight-ended arrows) and non-polar, slow migrating cells (square-ended arrows). Immunofluorescent staining of cells showed that 1 μM colchicine caused depolimerization of microtubules (**C**) as compared to control (see Fig 2A). The insert shows an enlarged region marked in the image. (**D, E**) Composite trajectories of 1 μM colchicine-treated cells under isotropic conditions (**D**) and in the presence of dcEF (**E**) show that cells exposed to dcEF moved towards the cathode, however with lower directionality comparing to colchicine-untreated cells (see Fig. 1D). Scale in μm.

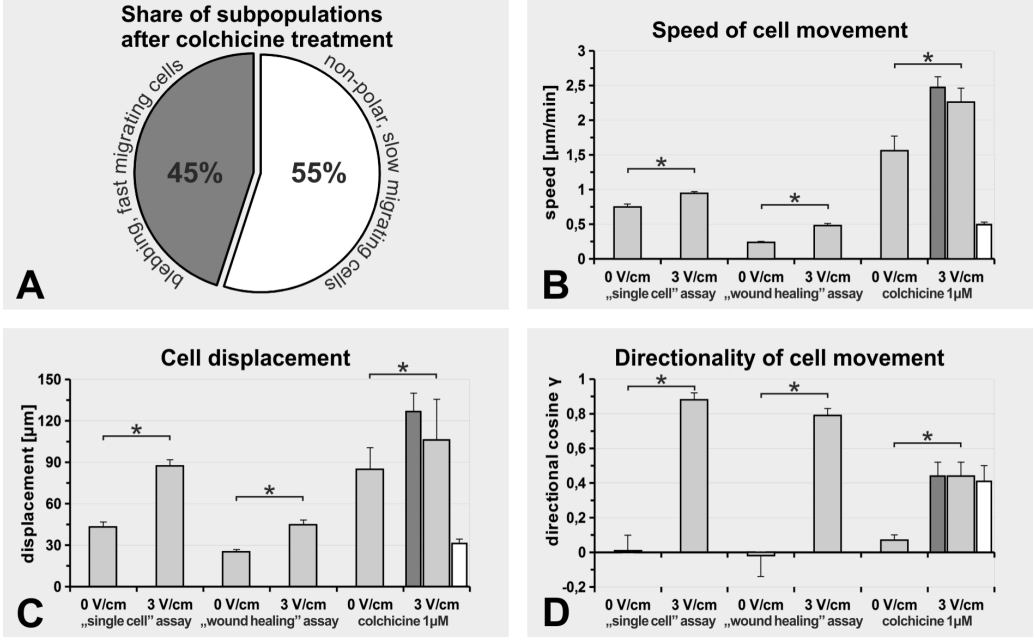


**Figure 4. Quantitative analysis of cell movement in isotropic conditions and in the presence of dcEF.**
(**A**) Diagram represents the percentage of two subpopulations of WC256 cells after 1 μM colchicine treatment. (**B, C, D**) Average values (grey bars) of parameters characterizing cell movement were quantified for cells in the absence or presence of dcEF in the "single cell", "wound healing" assays and after 1 μM colchicine treatment. Additionally, these parameters were estimated separately for blebbing (dark grey bars) and non-polar (white bars) WC256 cells exposed to dcEF after colchicine administration. (*) Statistical significance at $p<0.05$.

a crucial role in the directional movement of WC256 cells to dcEF, we investigated the effect of colchicine-stimulated depolimerization of MTs on electrotaxis of WC 256 cells. We confirmed that after colchicine treatment cells turned up into two subpopulations which differed in morphology and motile activity (Sroka *et al.*, 2002b). We observed that about 45% of cells formed blebs instead of lamellipodia and under the isotropic conditions migrated more effectively than cells moving in the absence of this agent (Fig. 3 and Fig. 4). Most of the remaining cells contracted and became rounded or transformed into non-polar cells and their translocation decreased two-fold compared to the control. After application of dcEF (3 V/cm) we analyzed cell movement of both subpopulations collectively and separately. We found that cells of both subpopulations moved towards the cathode however with lower directionality than under control conditions (cos $\gamma = 0.44 \pm 0.08$; $\gamma = 0.42 \pm 0.08$ and $0.87 \pm 0.08$ for blebbing cells; non-polar cells and colchicine-untreated cells, respectively) (Fig. 4). These results suggest that microtubules are not indispensable for electrotaxis of WC256 cells, however they may improve the directionality of their migration. It is also possible that formation of blebs at the leading edge instead of lamellipodia after colchicine treatment is responsible for the lower directionality of cell movement. On the other hand, the inhibitory effect of colchicine on electrotaxis of WC256 cells may be mediated by a different mechanism. We cannot exclude the direct effect of colchicine on membrane structure or its integrity, adhesion or ion channels, resulting in the disorganization of function. Although little is known about the mechanism of electrotaxis we believe that new experimental methods at the genome and proteome level will enable to elucidate it.

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